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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Process and Device for the Simultaneous Synthesis of Several Polypeptides

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Process and device for the fully automatic simultaneous synthesis of several polypeptides, in which up to 48 different polypeptides may be synthesided in an automatic pipette by the solid-phase method of synthesis. The device has individual reaction vessels for the synthesis of the individual polypeptides which are brought together to form one unit by a holding device. The simultaneous extraction of the fluids from the reaction vessels after each reaction or washing process takes place via the holding device.

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Process and device for the simultaneous synthesis of several polypeptides

For rapid evaluation of structure/activity equations on biologically active peptides by receptor binding studies and rapid determination of epitopes for immunology in peptides and proteins, relatively small amounts (less than 20 mg) of a plurality of peptides are required. These peptides are conveniently prepared by solid phase peptide synthesis. This synthesis is based on the method developed by R.B. Merrifield (G. Barany, R.B. Merrifield in The Peptides, Analysis, Synthesis, Biology, Vol. 2, 3-284 (1980), published by Gross, Meienhofer Academic Press, New York), in which the peptide chain is synthesised step by step. The synthesis steps can be summarised as follows:

a) Binding the first amino-acid of the peptide chain to a polymeric carrier via an anchor group,

b) condensing on the remaining amino-acids of the peptide chain step by step,

c) intermediate steps between the individual condensations, consisting of washing, cleaving any protective groups and neutralising,

d) if desired, acylating terminal amino groups,

e) cleaving the peptide from the carrier.

For this peptide synthesis a period of up to 18 hours, usually up to 4 hours per amino-acid must be allowed for the synthesis. (The individual condensations generally take one to two hours' reaction

time; between the condensations about 10 intermediate steps are generally required, each of which will require about 2-15 minutes). The preparation of peptides consisting of a large number of amino-acids is therefore very tiresome, labour-intensive and expensive.

A method for the solid phase synthesis of analogous peptides has been described by R.A. Houghten (Proc. Natl. Acad. Sci, USA, Vol. 82, pp. 5131-5135, August 1985, Immunology). According to this method the polymeric carrier for the synthesis is packed into small porous polypropylene bags in batches of 50-100 mg, the bags are sealed by fusion, the intermediate steps common to the syntheses (washing, neutralising, cleaving of protective groups) are carried out on all the bags simultaneously in a single reaction vessel and the individual condensations are carried out separately. The method may be carried out manually or partly automated using a peptide synthesizer.

The disadvantage of the method described is that the handling of the bags is rather laborious, the bags cannot be reused, the bags have to be separated from one another for the condensation of the different peptides and no control samples can be taken throughout the entire synthesis.

German Patent Application No. P 38 28 576.2 and the publication by G. Schnorrenberg and H. Gerhardt, Tetrahedron Vol. 45, No. 24, 7759-7764, 1989 describe a process and a device which allow a number of polypeptides to be synthesized simultaneously automatically and avoids the disadvantages mentioned above. The solid phase synthesis method mentioned above is modified so that it can be carried out with the aid of a suitably adapted pipetting robot. Hitherto, pipetting robots have been used for serial analysis. For example it is possible to use a pipetting robot made by TECAN, RSP 5052. Pipetting robots have the following external components: at least one arm having a metering

pipette, a clamp with storage vessels and a microtitre plate which may contain up to 96 wells. The robot arm brings the reagents from the storage vessels and places them in the respective wells in the microtitre plate and if necessary sucks liquids out of the wells. The cannula of the metering pipette may be constructed so that it is divided into two parts by a partition wall running from top to bottom. (By means of this partitioned cannula it is possible to supply two different metered amounts or to supply one metered amount and suck out one amount with one arm). The work pattern of the device is controlled by a computer programme. According to the above mentioned German Patent Application No. P 38 28 576.2 the solid phase peptide synthesis in a pipetting robot of this kind is carried out as follows:
carrier material (preferably granulated carrier material) is placed in the wells of a microtitre plate. The carrier material may be charged with the initial quantity of the desired peptide. The liquids required for the reactions and washing steps are kept in readiness in the storage vessels of the device. If at the end of the synthesis the peptide is to be separated from the carrier and/or if free amino groups are to be acylated, the reagents required for these reactions should also be kept in readiness in the storage vessels. The reaction times needed show that it is advisable to use a microtitre plate containing not more than 96 wells. Accordingly, a maximum of 96 different polypeptides can be synthesised in one programmed operation. In accordance with the programme, which is adapted to the synthesis of these peptides, the robot introduces the reagents and washing liquids into the individual wells and, after the required retention time, sucks out the supernatant liquid above the carrier. The process and the necessary adaptation of the device to the process is described more fully with reference to

the two-armed pipetting robot RSP 5052 made by Messrs TECAN. However, the application of the process is not restricted to this device. Pipetting robots of different constructions, especially one- or multi-armed robots may be adapted to the process according to the present invention. A microtitre plate having 96 wells is used. One well will contain, for example, 10 mg of resin which may be charged with an amino-acid, and will hold rather more than 300 µl of liquid. This quantity of resin corresponds to about 5 µmol of amino-acid or is suitable for the preparation of about 5 µmol of peptide. Conventional carrier materials based on polystyrene or polyacrylamide can be used. It is convenient to synthesize peptides containing not more than 20 amino-acids. The reagent solutions and washing liquids required for this are prepared in the storage vessels provided for this purpose. Arm 1 of the device is equipped with a metering pipette, arm 2 with a suction channel having a rinsing device. This rinsing device is preferably connected to a separately standing storage vessel for the solvent used. Synthesis is carried out in accordance with the programme in the connected PC. The arm 1 measures all the reagent solutions which are taken from open storage vessels. Before the metering pipette changes from one reagent solution to another, the metering pipette is rinsed with solvent in a special rinsing position. The arm 2 sucks up the reagent and washing liquids through a cannula fitted with a filter. In order to prevent resin losses and contamination of the adjacent wells the outside of this cannula is rinsed with solvent after each suction process by means of a line mounted on the side of the cannula. The next washing process is started at the same time with this solvent. The cannula is then rinsed in a cannula rinsing position. In order to separate the peptide from the resin, trifluoroacetic acid, for example, is introduced into the wells via the arm 1. After it has

been split off, the solution is sucked up with the suction cannula and transferred into a second microtitre plate from which it is then worked up.

The device described makes it possible to carry out automatic simultaneous synthesis of a number of polypeptides. One unsatisfactory feature, however, is that the liquids cannot be sucked up entirely and that it takes a relatively long time to suck up the reagents and washing liquids. The present invention overcomes these drawbacks. Moreover, it is possible to synthesize up to 25 μmol of peptide without changing the reaction vessels.

The present invention relates to an apparatus for simultaneously synthesizing a plurality of polypeptides by the solid phase synthesis method, comprising a plurality of reaction vessels and a holding device for the reaction vessels, these reaction vessels being open at the top and bottom, the bottom opening of the individual reaction vessels being covered by a filter, the retaining device being a closable vessel which has a possible connection for an inner gas feed line and a suction device and openings in which the reaction vessels are secured in such a way that their upper openings are accessible from above for the addition of the liquids required in the synthesis process whilst the bottom openings thereof are connected to the interior of the holding device.

This device may be operated in conjunction with the pipetting robot described above, for example.

The invention further relates to a process for the simultaneous synthesis of a plurality of polypeptides by the solid phase synthesis method using the apparatus described above, in which polymeric carrier material or polymeric carrier material charged with the first amino-acid or a peptide is placed in the reaction vessels, then the peptides are synthesized in the reaction vessels in accordance with the solid phase synthesis

method which is known per se and, if desired, free amino groups and/or hydroxy groups of the peptides are acylated and/or the peptides are subsequently separated from their carrier material, the reagents or washing liquids required for the individual steps being introduced into the reaction vessels by one or more robot arms with cannulas from the corresponding storage containers and after the required retention time of the reagents or washing liquids the liquids contained in the reaction vessels above the filters are simultaneously sucked out through the holding device, the individual steps of the process being controlled by the programme in the computer attached to the robot. In a preferred embodiment, inert gas is piped into the holding device throughout the entire synthesis process, with the exception of the phases in which the liquids are sucked out, and this inert gas is kept under such low pressure that it only prevents the liquids from seeping through the filters in the reaction vessels.

In a preferred embodiment of the device the holding device consists of a tub-shaped vessel with a plate-like cover, this cover having openings each of which holds a reaction vessel.

The holding device and reaction vessels are conveniently made from glass, metal (preferably stainless steel), polypropylene, teflon or polyamide 66 or a combination of these materials.

The retaining device and reaction vessels must be matched to one another so that the reaction vessels fit securely in the holding device. This may be achieved, for example, by screwing or fitting the reaction vessels into the holding device.

In a preferred embodiment of the device the cover of the holding device is provided with suitable conical openings and is preferably made of teflon or polyamide 66. The reaction vessels are cylindrical glass containers which have at the bottom a ground glass

section which is inserted in the holding device. The filters which cover the bottom openings in the reaction vessels are fritted glass or teflon filters. (It is advantageous to use reaction vessels which have a bore in the bottom into which the fritted teflon filter can be placed or pressed. After the end of a cycle of synthesis these fritted filters can be replaced by new ones).

When the apparatus according to the invention is operated with the pipetting robot mentioned above it is advisable to construct the apparatus with not more than 48 reaction vessels.

However, the application of the process is not restricted to this apparatus. Pipetting robots of different constructions, particularly one-armed or multi-armed robots, may also be adapted to the process according to the invention.

In order to carry out the process according to the invention, 10-50 mg of resin, for example, are placed in each reaction vessel. This quantity of resin corresponds to about 5-25 μmol of amino-acid or is suitable for preparing about 5-25 μmol of peptide. Conventional carrier materials based on polystyrene or polyacrylamide may be used. It is advisable to synthesize peptides containing not more than 30 amino-acids. The reagent solutions and washing liquids required for this are prepared in the storage vessels provided for this purpose. The arm of the pipetting robot is fitted with a metering pipette. Synthesis is carried out in accordance with the programme in the attached PC. In this way all the reagent solutions are metered in, having been taken out of the storage vessels sealed with teflon partitions. Before the metering pipette changes from one reagent solution to another, it is rinsed with solvent in a special rinsing position. The washing liquids required are introduced analogously. The removal of the desired peptide from the carrier

using trifluoroacetic acid may similarly be carried out automatically or manually.

The sucking out of all the liquids from the reaction vessels for the particular reaction or the washing steps is carried out simultaneously in all the reaction vessels by means of the suction device attached to the holding means (eg. water jet pump or diaphragm pump).

Generally, DMF or N-methylpyrrolidone is used as solvent. Accordingly, the reaction vessels and any rinsing device provided must be made from solvent-resistant material, eg. glass, polypropylene or teflon. In the standard commercial equipment the metering pipettes are made from stainless steel. This material is suitable for the process according to the invention.

The process is carried out, for example, with the following means (quantity per well): starting material is 10 mg of resin charged with Fmoc amino-acids (particle size 200-400 mesh); Fmoc-protected amino-acids are used in an excess of up to 10 times for each individual coupling step, ie. 200 µl of a DMF solution of 50 µmol Fmoc amino-acid and 50 µmol of 1-hydroxybenzotriazole and 100 µl of a DMF solution of 75 µmol of N,N-dicyclohexyl-carbodiimide are added; the coupling time is about 1 hour. The Fmoc protecting group is split off by means of 300 µl of a 40% solution of piperidine in DMF. The cleaving time is about 20 minutes. The washing steps are each carried out with 300 µl of DMF.

The acylation of free groups (NH_2, OH) may be carried out analogously by adding suitable acid anhydrides, eg. acetanhydride and pyridine. After these reactions have ended the solution is suction filtered and taken for processing.

The finished peptide can be removed from the carrier in the reaction vessels by the manual addition of trifluoroacetic acid (20 minutes' reaction time).

The peptides are isolated separately. As will become clear from the above explanation, all the steps of the peptide synthesis are carried out in open vessels. The method of synthesis according to the invention nevertheless produces the peptides in a very high degree of purity.

Figures 1 to 3 diagrammatically show an example of the apparatus according to the invention.

Figure 1 shows the holding device consisting of a tub (2) and cover (1). The tub (2) can be firmly closed by means of the cover (1), preferably by screw closures which screw the flange of the tub to the cover. The cover (1) has openings (3) which are arranged over the entire surface at regular intervals. (The drawing shows only some of the openings). The reaction vessels (4) are inserted in these openings. If the process is carried out in a number of reaction vessels which is less than the number of openings in the cover, the unused openings are sealed off by means of ground glass stoppers.

Two connections (5) and (6) are provided in the wall of the tub (2). Connection (5) is attached to the suction device and connection (6) serves for the supply of inert gas.

Figure 2 shows a cross section of a preferred embodiment of the holding device. This contains a guide vane (7) which is mounted like a second base in the tub (2), just below the connection (5). It is arranged to slope slightly towards the connection (5), so as to allow all the liquids sucked out to drain away entirely.

Figure 3 shows a diagrammatic view of a reaction vessel (4). The upper part of the vessel is cylindrical. The lower part of the vessel tapers and is fixedly connected to a ground glass section (9). The bottom opening in the reaction vessel is covered by a fritted glass or teflon filter (8).

In this embodiment, the tub (2) is made of metal

(preferably V2a steel), the cover (1) is made of teflon or polyamide 66 and the reaction vessels (4) are made of glass. The openings (3) are conical and correspond to the precise size of the ground glass sections (9) of the reaction vessels. The filters (8) are fritted glass filters G2 or G3 or fritted teflon filters made by G.T. Baker Chemikalien, DE-6080 Groβ-Gerau, order number 7329/03. If the apparatus is used together with the pipetting robot made by TECAN, RSP 5052, the tub (2) is conveniently produced with dimensions of about 165 x 127 x 45 (mm). The reaction vessels then have a total height of about 70 mm, of which about 25 mm consists of the cylindrical part above the fritted glass filter. The diameter of this upper part is 13 mm. It is convenient to construct the apparatus for not more than 48 reaction vessels.

The holding device is connected via connection (5) to a suction device, eg. a diaphragm pump having a 5 litre suction bottle provided in front of it. The holding device is also connected to an inert gas supply (eg. nitrogen bottle) via the connection (6). A pressure relief valve (usually set to about 0.1 bar) is provided in this line. Adjusting means are also provided in the lines, adjustable by means of a PC, for example.

An alternative embodiment of this device is constructed so that the tub (2) has only one connection which is connected to the two lines (inert gas/suction means) by means of a PC-controllable 3-way valve.

The example which follows illustrates the course of the process according to the invention: the apparatus described above is used in conjunction with a pipetting robot. The process is computer-controlled. Carrier material already charged with a protected amino-acid or a short peptide is placed in the reaction vessels.

Part of the synthesis cycle:

Step		Operation
1	Valve N_2 open, vacuum shut	
2	Metering of DMF 3 min.	Washing
3	Valve N_2 shut, vacuum open	Suction
4	Valve N_2, open, vacuum shut	
5	Metering 40% piperidine in DMF 3 min.	Cleaving of protective groups
6	Valve N_2 shut, vacuum open	Suction
7	Valve N_2 open, vacuum shut	
8	Metering 40% piperidine in DMF 20 min.	Cleaving of protective groups
9	Valve N_2 shut, vacuum open	Suction
10	Valve N_2 open, vacuum shut	
11	Metering of DMF 30 sec.	Washing
12	Valve N_2 shut, vacuum open	Suction
13	Valve N_2 open, vacuum shut	
14-40	Repetition of steps 11-13	Washing 9x
41	Metering of desired Fmoc-amino-acid/HOBt in DMF	1st coupling
42	Metering of DIC/DMF 40 min	1st coupling
43	Valve N_2 shut, vacuum open	Suction
44	Valve N_2 open, vacuum shut	
45-48	Repetition of steps 41-44	2nd coupling
49-78	Repetition of steps 11-13	Washing 10x

Compared with the process and apparatus according to the German Patent Application No. P 38 28 576.2 referred to above, the apparatus according to the invention substantially reduces (approximately by half) the time taken for the synthesis cycle. The synthesis is reliable and clean since the liquids are sucked up completely. The course of the synthesis can easily be monitored as the reaction vessels are open.

Claims

1. Device for the simultaneous synthesis of several polypeptides by the solid phase synthesis method which comprises a plurality of reaction vessels and a holding device for the reaction vessels, the reaction vessels being open at the top and bottom, the bottom opening of the individual reaction vessel being covered by a filter, the holding device being a closable vessel which has a possible connection for an inert gas feed line and a suction device as well as openings in which the reaction vessels are secured so that their top openings are accessible from above for the addition of the liquids needed in the synthesis process whilst the bottom openings are connected to the interior of the holding device.

2. Apparatus according to claim 1, characterised in that the holding device consists of a tub-shaped vessel with a plate-like cover, the cover having openings each of which holds a reaction vessel.

3. Apparatus according to claim 1 or 2, characterised in that the reaction vessels are cylindrical glass containers which have at their lower end a ground glass section which is inserted in the holding device.

4. Apparatus according to one of claims 1 to 3, characterised in that the filters which cover the bottom openings of the reaction vessels are fritted glass filters or fritted teflon filters.

5. Apparatus according to one of claims 1 to 4, characterised in that the liquids needed for the synthesis process are introduced into the reaction vessels by means of a pipetting robot.

6. Process for simultaneously synthesizing a plurality of polypeptides using the solid phase synthesis method using the apparatus according to one of claims 1 to 5, wherein polymeric carrier material or polymeric carrier material charged with the first amino-acid or a peptide is placed in the reaction vessels, then the peptides are synthesized in the reaction vessels according to the solid phase synthesis method which is known per se and, if desired, free amino groups and/or hydroxy groups of the peptides are acylated, the reagents or washing liquids required for the individual steps being introduced into the reaction vessels from the corresponding storage containers by means of one or more robot arms with cannulas and, after the required retention time of the reagents or washing liquids, the liquids contained in the reaction vessels above the filters are sucked out simultaneously through the holding device, the individual steps of the process being controlled by the programme in the computer which is connected to the robot.

7. Process according to claim 6, characterised in that throughout the entire synthesis process, with the exception of the phases in which the liquids are sucked out, inert gas is piped into the holding device which is kept under such a low pressure that it prevents the liquids from seeping through the filters in the reaction vessels.

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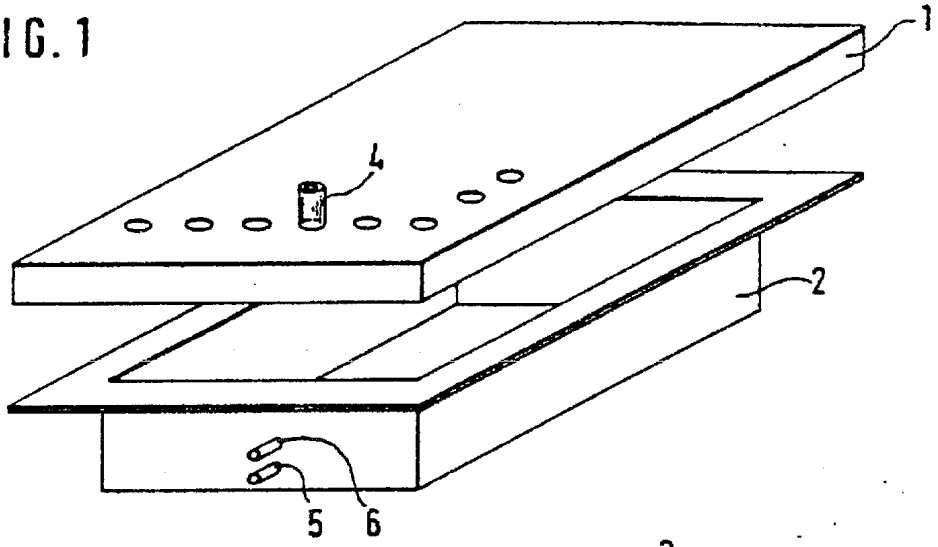
FIG.1



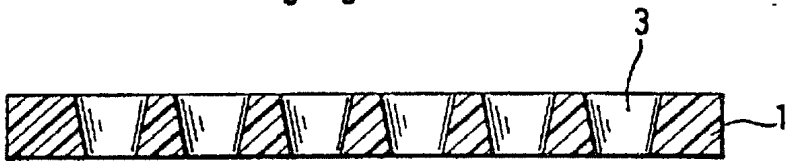


FIG.2

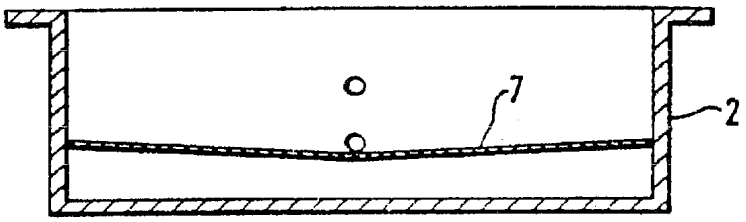
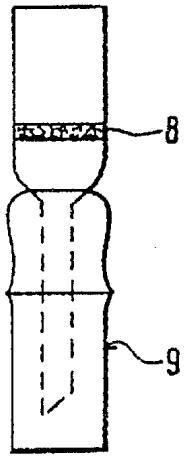


FIG.3



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